NOTES ON THE PRECISION OF A MODIFIED ROUTINE NITRATE-NITRITE ANALYSIS

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ABSTRACT

Factors responsible for difficulties in using the strychnidine method for an analysis of nitrate-nitrite content of sea water have been investigated. It has been demonstrated that their effects can be minimized and that the ease of analysis is materially increased by certain modifications, one of which is the adjustment of the concentration of nitrate by dilution to bring the optical density within the best working range of the photometer used. The largest single source of error was found to be variation in the rate of heat formation during the mixing of the sample and reagent, which could be minimized by following the mixing procedure recommended. It is believed that this work offers improved precision without affecting the value of the technique as a rapid routine method for large-scale investigations.

In using the strychnidine method for analysis of nitrate-nitrite in sea water, we experienced many of the same difficulties encountered by other operators. In an effort to minimize these difficulties, a modified method has been developed which is now employed in the laboratory of the Gulf Fishery Investigations. Actually, it is a modified version of the analysis prescribed by Zwicker and Robinson.[1]

This modification controls color densities of the reagent-sample mixture so that the range of concentrations found in sea water can be determined by using 3 ml micro cells (cylindrical cell 6.75 cm long and 1.19 cm inside diameter) in a Fisher A. C. Electrophotometer. The mixture is obtained by diluting the water sample with two parts of distilled water and then adding this diluted sample to an equal volume of a reagent consisting of 0.3 millimole of strychnidine per liter of concentrated sulfuric acid.

[1] Zwicker, B. M. G. and R. J. Robinson. 1944. The photometric determination of nitrate in sea water with a strychnidine reagent. J. Mar. Res., *5:* 214–231. They used the Zeiss-Pulfrich photometer equipped with 0.1–15 mm variable depth cell. Their procedure was as follows:

Equal volumes of reagent and sample were carefully mixed in aged pyrex tubes by four careful but rapid transfers from one to the other. The hot mixtures were immediately placed in the dark for three to five hours. Measurements were then made with a photometer.

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This reagent is suitable for determining concentrations found in waters which range from 0 to about 45 microgram atoms of NO_3-NO_2 nitrogen/liter (μg-at NO_3-NO_2-N/l). The -log T readings for this range cover practically the entire scale of the electrophotometer and at the same time permit the use of a calibration curve having the best possible slope. Thus, maximum efficiency of the photometer is achieved. At concentrations above 45 μg-at/l, the slope flattens too much for use.

The dilution of samples does not necessarily mean that all errors will be increased proportionally. Actually, the dilution moves the concentration of many samples to a more sensitive region of the NO_3-NO_2 calibration curve.

Zwicker and Robinson recommended that the strength of the reagent and the depth of the cell be dependent upon the range of NO_3-NO_2 concentration in the sample in order to utilize the optimum operating conditions of reagent and photometer.[2] In our routine sampling of hundreds of water samples having the range of NO_3-NO_2 concentration found in the Gulf, their recommendation would be impractical since it would necessitate a preliminary investigation of all samples to approximate the magnitude of the NO_3-NO_2 concentration.

A chemist and his helper can easily run 200 determinations in an eight-hour period when employing our modification, which is as follows:

1. With a 1 ml pipette, transfer 1 ml of sample which has had no preliminary preparation, such as filtration, etc., into each of two 10 ml pyrex test tubes (for duplicates).
2. With a 3 ml automatic pipette, add 2 ml of distilled water to each of the 10 ml tubes.
3. Using a 5 ml automatic pipette, add 3 ml of strychnidine reagent to each tube. To prevent boiling during this addition, tilt the tube to about a 45° angle and allow the reagent to run down the inside so that it forms a layer under the water sample. It is important that this step be performed with great care to avoid unnecessary mixing of reagent and sample.
4. With the aid of a 15 ml pyrex tube, mix by gently pouring the contents of each tube into the mixing tube and back again only once. It is suggested that about 15 mixing tubes be used in rotation and that each tube be allowed to drain after use.

[2] Their recommended procedure for the Zeiss-Pulfrich photometer follows:

NO_3 *range* (μg-at/l)	*Strychnidine* (millimoles/l)	*Sample* (ml)	*Size of Test Tube* (mm)	*Standing Time* (hours)	*Thickness of Cell* (stratum, mm)
0.2–4	0.10	14	20 ×155	4–24	50
2.5–10	0.10	3	13 ×100	2–28	10
5–15	0.50	3	13 ×100	2–5	5
1.2–40	1.0	3	13 ×100	2–5	2
2.5–75	5.0	3	13 ×100	2–4	1

5. Store samples in darkness for four hours.
6. Check color density with the Fisher A. C. Electrophotometer by using 3 ml micro cells and 525 mμ "B" filter.
7. Check the results against standard controls which are run with the samples and which are interspersed among them during the reading of color densities.

ESTIMATE OF PRECISION OF THE METHOD

An estimate of precision was obtained by running a series of checks within the range of values that are possible with a Fisher A. C. Electrophotometer equipped with a 3 ml micro cell and with a reagent containing 0.3 millimole of strychnidine in a liter of concentrated sulfuric acid. This range includes concentrations from 0 to 15 μg-at/l of nitrate.

The range was covered in 1 μg-at/l steps. Approximately 50 replicate determinations were made on each of the 16 concentrations that were checked. Color density differences between samples and distilled water were read directly from the electrophotometer in -log T units after which they were converted to μg-at/l units by assuming a constant slope between two adjacent sets of standards. An estimate of precision for the concentrations tested is tabulated in column 4 of Table I.

TABLE I. Statistics Pertaining to NO_3-NO_2 Analyses at Various Concentration Levels

Concentration level	N	$\bar{X}$	S	l_1	l_2
0	55	0.000	0.1551	−0.0558	0.0558
1	52	1.004	0.1171	0.9606	1.0474
2	55	1.987	0.1156	1.9454	2.0286
3	52	2.988	0.1131	2.9461	3.0299
4	51	4.022	0.1640	3.9605	4.0835
5	56	4.993	0.2463	4.9052	5.0808
6	53	6.030	0.0799	6.0007	6.0593
7	55	6.980	0.2791	6.8796	7.0804
8	56	8.014	0.1939	7.9449	8.0831
9	53	9.002	0.3054	8.8899	9.1141
10	51	9.998	0.3465	9.8682	10.1278
11	43	11.040	0.2927	10.9197	11.1603
12	54	12.030	0.3937	11.8869	12.1731
13	53	13.028	0.3478	12.9003	13.1557
14	46	13.974	0.3022	13.8540	14.0940
15	57	14.974	0.3807	14.8396	15.1084

N—Number of replicates.
$\bar{X}$—Average of replicates in μg-at NO_3-NO_2-N/l.
S—Standard deviation.
l_1; l_2—Fiducial limits of $\bar{X}$ at a probability level of .01.

In the modified routine analysis, the precision would be approximately one-third that shown in column 4 of Table I because all samples are diluted with two parts of water; hence, the standard deviation of the sets would be increased by three. Also, the difference in error of the two methods due to volumetric difference would have to be included. This, as shown in Table II, is negligible. Based on these facts, Table III has been constructed to show the precision that can be expected from routine analysis.

TABLE II. PRECISION OF VOLUMETRIC MEASUREMENTS

No. 1 *Steps involved in routine analysis.*

1.—1 ml sample delivery using 1 ml pipette.
2.—2 ml H_2O delivery using 3 ml automatic pipette.
3.—3 ml reagent delivery using 5 ml automatic pipette.

Step	1	2	3
N	31	33	30
S^2	2.35×10^{-5}	6.36×10^{-5}	1.69×10^{-4}

$$S^2 \text{ due to volumetric deviation} = \frac{2.35 \times 10^{-5}}{6} + \frac{6.36 \times 10^{-5}}{3} + \frac{1.69 \times 10^{-4}}{2} = 1.096 \times 10^{-4}.$$

S due to volumetric deviation = .0105.

No. 2 *Steps involved in estimating precision.*

1.—3 ml sample delivery using 5 ml automatic pipette.
2.—3 ml reagent delivery using 5 ml automatic pipette.

Step	1	2
N	20	30
S^2	6.42×10^{-5}	1.69×10^{-4}

$$S^2 \text{ due to volumetric deviation} = \frac{6.42 \times 10^{-5}}{2} + \frac{1.69 \times 10^{-4}}{2} = 1.166 \times 10^{-4}.$$

S due to volumetric deviation = .0108.

N—Number of replicates. S^2—Variance. S—Standard deviations..

ESTIMATE OF PRECISION OF EACH STEP IN THE NITRATE DETERMINATION

The precision of the modified technique was estimated by determining the accuracy that could be expected from each individual step. Experiments were conducted in which the various steps of the technique were deliberately varied to considerable extents. The effects of these alterations on the final nitrate concentration were then ascertained.

Human and instrumental errors introduced in reading color density differences were estimated by measuring the deviation that occurred in reading sets of replicates from a series of standards. A standard was made up and a portion poured off and checked in the photometer.

TABLE III. STATISTICS PERTAINING TO ROUTINE NO_3-NO_2 ANALYSIS

Concentration Level	N	S^2	$\bar{X}$	S_1^2	S_2^2	$S^2-(S_1^2+S_2^2)$	l_1	l_2
0	55	0.2406	0.000	0.0036	0.0001	0.2369	−0.0558	0.0558
3	52	0.1234	3.012	0.0036	0.0001	0.1197	2.8818	3.1422
6	55	0.1202	5.964	0.0036	0.0001	0.1165	5.8362	6.0858
9	52	0.1153	8.964	0.0049	0.0001	0.1103	8.8383	9.0897
12	51	0.2423	12.066	0.0049	0.0001	0.2373	11.8815	12.2505
15	56	0.5461	14.979	0.0081	0.0001	0.5379	14.7156	15.2424
18	53	0.0574	18.090	0.0121	0.0001	0.0452	18.0021	18.1779
21	55	0.7013	20.940	0.0289	0.0001	0.6723	20.6388	21.2412
24	56	0.3385	24.042	0.1521	0.0001	0.1863	23.8347	24.2493
27	53	0.8394	27.006	0.0625	0.0001	0.7768	26.6697	27.3423
30	51	1.0854	29.994	0.1156	0.0001	0.9697	29.6046	30.3834
33	43	0.7720	33.120	0.1849	0.0001	0.5870	32.7591	33.4809
36	54	1.3912	36.090	0.2704	0.0001	1.1207	35.6607	36.5193
39	53	1.0952	39.084	0.3600	0.0001	0.7351	38.7009	39.4671
42	46	0.8217	41.922	0.2304	0.0001	0.5912	41.5620	42.2820
45	57	1.3003	44.922	0.3025	0.0001	0.9977	44.5188	45.3252

Concentration Level—Approximate Concentration in μg-at NO_3-N/l Tested.

N—Number of replicates.
$\bar{X}$—Average of replicates expressed in μg-at NO_3-N/l.
S^2—Variance.
S_1^2—Variance due to instrumental and operational deviation.
S_2^2—Variance due to volumetric deviation assuming all deviations are accumulative.
l_1; l_2—Fiducial limits of $\bar{X}$ at a probability level of .01.

The sample was then removed from the instrument and poured back into the original batch of standard. This cycle was repeated approximately 50 times for each concentration. Instrumental and operational errors were based on deviation in these sets of readings. The standards used for this check consisted of various dilutions of a nonfading red compound (red ink). This eliminated possible deviation due to color fading during the time required to read a set of replicates. The results, tabulated in column 5 of Table III are also presented in Fig. 1.

The errors involved in pipette deliveries result from variations in measuring: 1 ml of sample with a 1 ml pipette, 2 ml of distilled water with a 3 ml automatic pipette, and 3 ml of reagent with a 5 ml automatic pipette. The variation that can be expected with these three instruments was estimated by measuring equivalent amounts of distilled water for the 1 and 2 ml measurement and of sulfuric acid for the 3 ml reagent measurement, after which the accuracy of each measurement was determined by weight on an analytical balance having a sensitivity of approximately 1/10 mg.

The results of this check are given in Table II and in column 6 of Table III. The effect of deviations in sample and reagent measurements on the final nitrate concentration was determined by running several series of checks in which variations were deliberately intro-

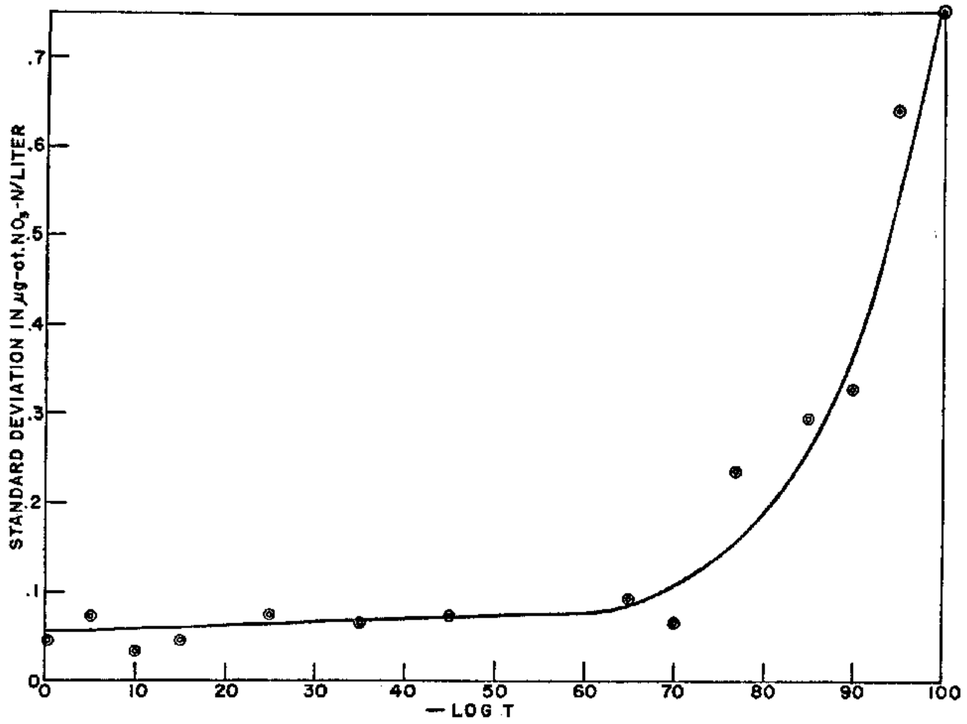

Figure 1. Deviation in NO_3 concentration caused by instrumental and operational errors.

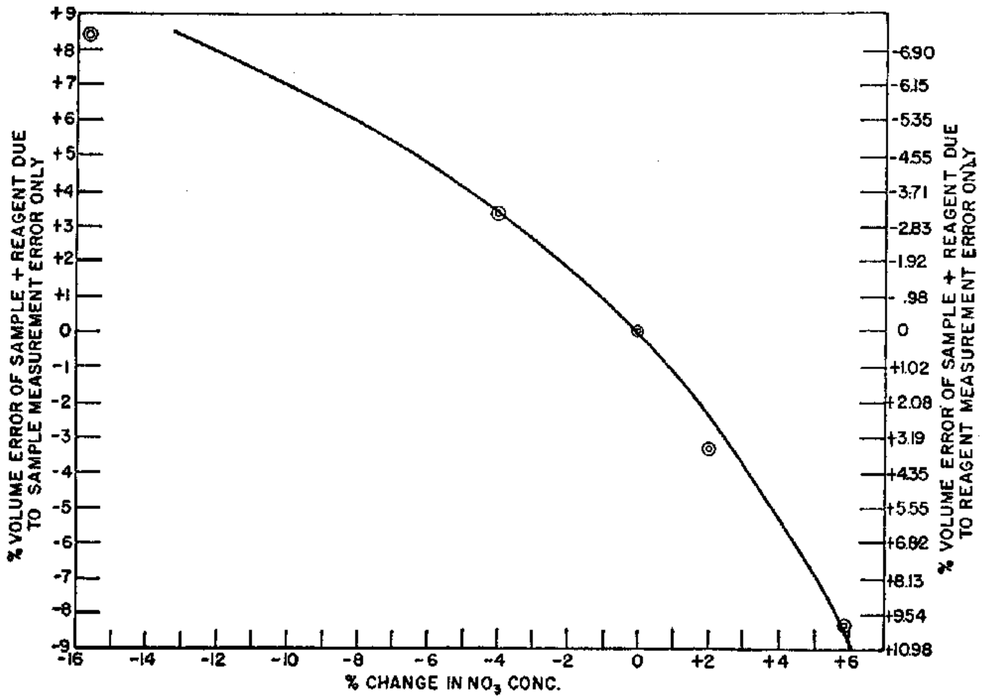

Figure 2. Per cent errors in NO_3 concentration caused by volumetric deviations.

duced. The results, which show variation in nitrate concentration vs. variations in sample and reagent measurements, are presented in Fig. 2.

We found that variation in the rate of heat formation during the mixing of the sample and reagent is the greatest single factor that affects the precision of the results. Investigation has shown that the degree of variation in color formation of replicate samples is dependent on the rate of removal of the heat produced during the mixing opera-

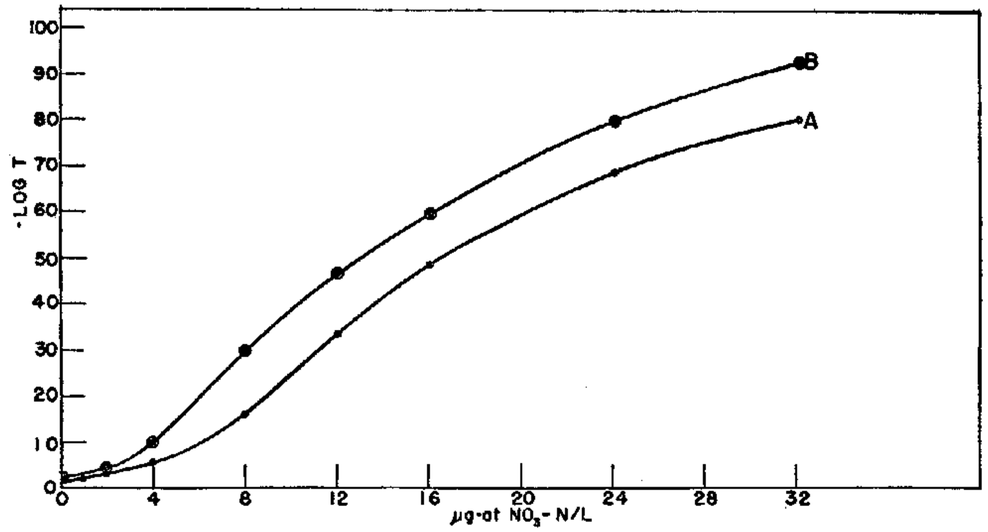

Figure 3. NO_3 calibration curve showing effect of rate of heat formation on color development. *A*—high rate of heat formation. *B*—low rate of heat formation.

tion. A high rate of increase in temperature results in a minimum color density, whereas a low rate of increase results in a maximum color density. By standardizing the mixing operation, it has been possible to reduce greatly the variability due to heat, thereby increasing the precision of the method.

The effects of high and low rates of heat formation are demonstrated in two calibration curves, *A* and *B* of Fig. 3. Both curves were made from a single set of standard samples and from the same batch of reagent. The determination of values for the two curves was based on the average of 10 replicates run alternately to minimize errors due to time difference. A maximum rate of heating was obtained by mixing the samples and reagent rapidly (see Curve *A*). A minimum rate was obtained by carefully adding reagent to sample in such a way that two distinct layers were formed. These were then mixed by pouring them into a "mixing" tube and back into the original tube; thus, the mixture was poured only twice, from original tube to mixing tube and back again.

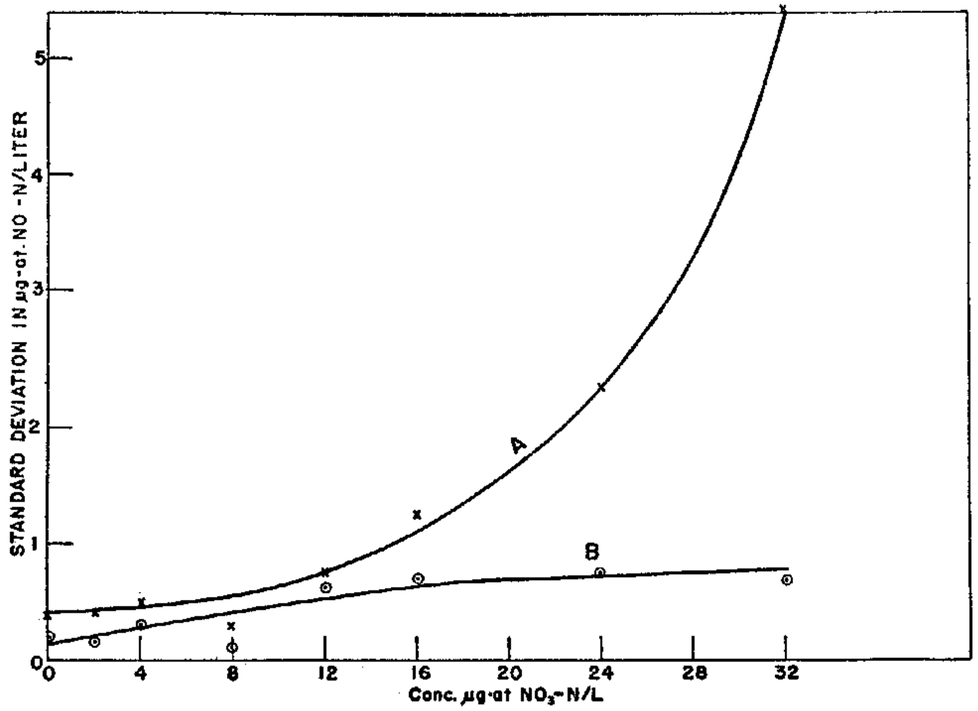

Figure 4. Comparison of standard deviations at various NO_3 concentration levels: *A*, when rate of heat formation during the mixing of reagent and sample is high; *B*, when the rate of heat formation is low.

The range of values represented by the horizontal distance between curves *A* and *B* represents the deviation that could occur within a set of replicate samples if an operator were unfamiliar with the relationship that exists between rate of heat formation and color formation. A comparison of the standard deviation of the two sets is shown in Fig. 4.

The low degrees of variation exhibited by *B*, as compared to *A*, indicate that careful mixing is necessary if good precision is to be maintained.

DISCUSSION

The entire check on precision of the method could not be completed during one session. Had it been possible, then the average of the 16 sets could have been used in the construction of a calibration curve, thus eliminating the necessity of arriving at slope values by assuming straight line relationships between two sets of values. The fact that all 16 points do not form a smooth curve emphasizes the importance of including a set of standards with every run of samples. We found that there was considerable day-to-day variation in the density of color formation, probably due to temperature difference, methods of

mixing sample and reagent, and other conditions that would be difficult to control.

A four-hour color development time was allowed for all determinations. This time was based on a check of color density vs. time for concentrations of 0, 6, 18, 25, 30, and 45 µg-at NO_3-N/l. Results show that rate of development at 30° C became minimal between the fourth and fifth hour. The room temperature during the experiments was approximately 30° ± 2° C.

The final nitrate concentration varies almost directly with the deviations normally expected in the measurement of sample, reagent, and distilled water. This is illustrated in Fig. 2, in which volumetric errors have been deliberately introduced. This direct relationship was assumed to be the case.

A large percentage of the total deviation incurred in the modified technique cannot be accounted for by instrumental, operational, or volumetric errors. The data in columns 3 and 7 of Table III demonstrate that variation in rate of heat formation during the mixing of sample and reagent probably accounts for a large percentage of this difference even though efforts were made to minimize this effect.